Journal of Medical Virology 54:92–94 (1998)

Titration of Recombinant Woodchuck Hepatitis Virus DNA in Adult Woodchucks

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In vivo transfection of Eastern woodchucks (*Marmota monax*) with recombinant woodchuck hepatitis virus (WHV) DNA is effective in inducing virus infection for the study of replication, pathogenicity, and oncogenicity of wild-type and mutated WHV. The one drawback to this procedure is the need for preparation of large amounts of WHV DNA. Reduction of the amount of WHV DNA in the transfection protocol necessary to induce infection would save considerable time and resources. Therefore, we conducted a titration of WHV DNA, ranging from 50 μg to 50 pg of DNA, in adult woodchucks to determine the minimum infectious dose of recombinant WHV DNA. As little as 50 ng of transfected WHV DNA induced productive infection in adult woodchucks. Thus, transfection with large amounts of recombinant WHV DNA appears to be unnecessary. ***J. Med. Virol. 54:92–94, 1998.***
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KEY WORDS: Woodchuck hepatitis virus infections; DNA; titration transfection; in vivo

INTRODUCTION

Hepatitis B virus (HBV) chronically infects over 300 million individuals worldwide. These individuals are at a high risk for developing liver cirrhosis and hepatocellular carcinoma (HCC) [Beasley, 1988; Beasley et al., 1981; Koike et al., 1989]. Formerly, the study of the biology of HBV infection was hampered by the lack of an appropriate animal model because of the species specificity of the virus (only higher primates such as chimpanzees are susceptible to infection). However, experimental infection of the Eastern woodchuck with woodchuck hepatitis virus (WHV), a natural pathogen of this species, has proven to be an excellent model system for the study of many aspects of HBV replication and pathogenesis [reviewed in Cote and Gerin, 1996]. Specifically, the WHV and HBV genomes share 65% nucleotide sequence homology and possess a similar organization [Galibert et al., 1982; Miller et al., 1989]. Furthermore, WHV infection in woodchucks usually results in acute hepatitis, progressing to chronicity, similar to HBV infection in humans [Cote et al., 1991; Girones et al., 1989a; Korba et al., 1989; Miller et al., 1990]. Also, hepatocellular carcinoma (HCC) develops in ~2–4 years in animals chronically infected with WHV [Gerin et al., 1989; Popper et al., 1987] as compared to 20–30 years in humans infected chronically with HBV.

The study of the biology of WHV infection in woodchucks has been part of a long-term collaborative effort [Cote and Gerin, 1996; Gerin et al., 1989; Hornbuckle et al., 1985; Korba et al., 1989; Miller et al., 1990; Popper et al., 1987]. In addition to standard intravenous inoculation of infectious serum, the following techniques have been developed for inducing WHV infection in woodchucks: (1) transfection of adult woodchucks by injecting the surgically exposed livers with recombinant WHV DNA; (2) transfection of neonatal woodchucks by injecting the liver percutaneously with recombinant WHV DNA; and, (3) infection of neonatal woodchucks with infectious WHV by subcutaneous injection. Recently, we conducted a series of in vivo woodchuck transfection studies using recombinant WHV DNAs [Chen et al., 1993; Chen et al., 1992; Girones et al., 1989a; Girones et al., 1989b]. Transfection of adult woodchucks with a standard inoculum of 50 μg of monomeric, recircularized wild-type WHV8 genomes re-

Contract grant sponsor: National Institute of Allergy and Infectious Diseases; Contract grant numbers: N01-AI-72623; N01-AI-82698.

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Accepted 8 September 1997

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sulted in an infection rate of 100% [Girones et al., 1989a]. One drawback in using recircularized WHV DNA is the preparation of large amounts of the monomeric WHV DNA. Therefore we wished to determine the minimum dose of WHV cDNA that would initiate infection reproducibly in woodchucks following intrahepatic transfection.

MATERIALS AND METHODS

Preparation of the monomeric, re-circularized WHV DNA used in these studies was accomplished by propagation of bacteria containing the relevant construct, purification of plasmid DNA, digestion with a restriction endonuclease to release vector and viral sequences, gel purification of WHV sequences, and recircularization of WHV DNA under dilute conditions (~5 μg/ml) to optimize production of monomeric, circular genomes. During the entire procedure extensive precautions were taken to prevent contamination of the DNA preparations by other recombinant WHV genomes.

Serial ten-fold dilutions of monomeric, recircularized WHV8 DNA were made in sterile phosphate-buffered saline (PBS) and stored at −80°C until use. Carrier DNA was not added since the quantity of DNA involved represented a significantly large number of genomes (~3×10^{11} WHV genomes per μg of DNA) and we wished to avoid the risk of recombination between viral and carrier sequences as well as any problems related to interference or inhibition of replication of WHV caused by the carrier molecules. The surgically exposed livers of adult woodchucks were inoculated with the DNA solution using a sterile 1.5 inch, 27 gauge needle and a 1 ml insulin syringe. Each dilution of WHV DNA in PBS was thawed at 4°C and used for injection without any further treatment. Injection of 100 μl of the DNA solution was performed at five independent sites on the liver for a total volume of inoculum of 0.5 ml per woodchuck. After each injection the needle was held in place for 15 sec and then slowly removed to reduce backflow and to allow the DNA solution to diffuse into the hepatic tissue.

The status of WHV infection in transfected woodchucks was monitored bi-weekly using serological assays (ELISA or RIA assays) for WHV surface antigen (WHsAg) and for antibodies against the surface (anti-WHs) and core (anti-WHc) proteins [Cote et al., 1993]. A woodchuck was considered infected with WHV when any of these markers became positive. Twenty adult woodchucks were used in this study. Three woodchucks were inoculated with each dose of recombinant WHV DNA with one exception: only two animals received the 50 μg DNA dose.

RESULTS

The time lapsed between transfection and the first appearance of serological evidence of WHV infection was 8–12 weeks in woodchucks receiving all but the lowest infectious dose of WHV DNA. The results revealed that while 50 μg, 5 μg, 500 ng, and 50 ng of WHV DNA were capable of inducing infection in all animals tested, 5 ng of the same preparation infected only two of three woodchucks. Woodchucks that received either 500 pg or 50 pg of WHV DNA failed to develop markers indicative of infection during the two years following inoculation (Table 1). Thus, the 50% woodchuck infectious dose is ~1 ng. There was no significant variation in the time to appearance of serological markers of viral infection in any of the animals transfected with 50 ng–50 μg of WHV DNA. However, for the two animals that were transfected with 5 ng of WHV DNA and became infected, the time to appearance of seroconversion was 16 weeks. All infected woodchucks had unequivocal serologic markers of infection and all uninfected animals were completely negative for serologic markers. The results indicate that 50 ng of transfected WHV DNA is the minimum amount of DNA necessary for inducing productive WHV infection reproducibly in adult woodchucks. Such amounts of monomeric, recircularized WHV DNA can be prepared easily, thus eliminating the need for the purification of larger amounts of recombinant WHV DNA.

TABLE I. Transfection of Adult Woodchuck Livers with WHV DNA

Amount of WHV8 DNA	Woodchuck number	Infection status
50 μg	2741	+[a]
	2960	+
5 μg	2402	+
	2766	+[a]
	2953	+
500 ng	894	+[a]
	2539	+[a]
	3017	+
50 ng	2157	+
	2517	+[a]
	2730	+
5 ng	2450	+
	2983	+
	3243	−
500 pg	937	−
	2767	−
	3602	−
50 pg	942	−
	2764	−
	3214	−

[a]Woodchuck developed a chronic infection.

DISCUSSION

Historically, transfection of recombinant DNA into cells was achieved by calcium phosphate precipitation of DNA onto cell membranes [Graham and Van der Eb, 1973] or uptake was facilitated by DEAE-dextran [McCutchan and Pagano, 1968]. A successful direct transfection of plasmid DNA into the liver or spleen of mice was reported by Dubensky and colleagues using a modified calcium phosphate precipitation method and 10 μg of recombinant DNA per mouse [Dubensky et al., 1984]. Our results demonstrate that direct injection of an amount of recombinant DNA 200-fold less (i.e., 50 ng) and without special treatment (e.g., calcium phos-

phate precipitation, addition of DEAE-dextran, etc.) is sufficient for the successful transfection of 100% of the animals. Similarly, Will et al. [Will et al., 1982] successfully transfected chimpanzees with HBV cDNA without special treatment such as calcium phosphate precipitation but their inoculum also contained more than 10 ug of cDNA, at least 2,000 times the minimum infectious dose in the present study. In addition, we found that transfection with 50 or 500 pg of WHV DNA (~1×10^7 or 1×10^8 genomes, respectively) did not produce infection of woodchucks as measured in our assay. Importantly, the latter finding suggests that minute amounts (i.e., several genome copies) of contaminating DNA should not be a factor in animal transfection experiments designed to evaluate the replication competence and biology of WHV mutants. Overall, this study demonstrates that the successful transfection of woodchucks can be accomplished reproducibly with a relatively small amount of recombinant viral DNA. Application of this procedure to other viruses and laboratory animals may be useful for future experiments.

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